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Borna disease virus antigen distribution in naturally infected bicolored white-toothed shrews, *Crocidura leucodon*, supporting its role as reservoir host species

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1. ABSTRACT

Borna Disease is a severe, immunopathological disorder of the central nervous system, caused by infection with Borna Disease Virus (BDV). The main known naturally affected animal species in endemic areas in central Europe are horses and sheep[24].
In this study we present evidence of shrew mice *(Crocidura leucodon*) as a vector of BDV. The widespread presence of viral antigen and –RNA in the organs of this animal species without producing pathological lesions differs from the classical hosts such as equines, small ruminants and other domestic animals naturally infected with BDV.
The detection of BDV in the shrew mice was achieved by immunohistochemistry and by TaqMan® Real Time RT-PCR. RT-PCR amplification products were sequenced, and the sequences were compared with those from horses and sheep originating from the same geographical region, which had died from Borna Disease (BD).

2. INTRODUCTION

Borna Disease Virus (BDV) is the causative agent of Borna disease (BD), a mostly fatal meningoencephalitis originally detected among horses of Germany, Switzerland and the Principality of Liechtenstein. Natural hosts of BDV are horses, sheep and other farm animals. Many other warm-blooded vertebrates ranging from rodents to non-human primates are susceptible to experimental infection with BDV. In these animals, BDV infection may either remain clinically inapperent, or it may lead to severe neurological abnormalities and eventually to death. Numerous studies with experimentally infected rats and mice have conclusively demonstrated that BD is caused by immunopathological mechanisms in which the antiviral CD8 T cell response results in neurological disorder[3, 18, 20, 21].

2.1. History

Borna Disease was first seen in 1976 in sheep in Switzerland. Since then there were sporadically cases of this disease concerning sheep and horses. The first reports however describing typical clinical characteristics of BD date back several hundred years. The clinical signs were described as pain which pushes the horses towards walls or makes them dull and dumb.

The disease became most evident and important in 1895, when a large number of horses of a cavalry regiment in the town of Borna in Saxony near Leipzig became severely ill and showed typical symptoms of a severe central nervous system disease. After this outbreak the disease was called Borna Disease.

Joest and Degen characterized the histopathology of BD and demonstrated that the disease was actually related to severe inflammatory reactions. Furthermore, they discovered the pathognomic intranuclear inclusion bodies, named Joest-Degen bodies[20].

2.2. Etiology

2.2.1. Taxonomy and morphology

BDV represents the only member of the new virus family *Bornaviridae* within the order *Mononegavirales* (table 1). Electron microscopic studies of negative-stained cell-free

BDV infectious particles have shown that they are of spherical morphology with a diameter ranging from 70 to 130 nm. These particles contain an internal electron-dense core (50 to 60 nm) and a limiting outer membrane envelope, which appears to be covered with spikes approximately 7.0 nm long. Virus infectivity is rapidly lost by treatment at 56°C, as well as at pH's below 5 and above 12, and by treatment with organic solvents, detergents, formaldehyde and exposure to ultra violet radiation. There are four known virus strains: Borna V, Borna HE/80, BornaNo/98 and Borna H1766. BDV is characterized by an extraordinary high sequence conservation otherwise uncommon for RNA viruses.

Table 1. Taxonomic structure of the order Mononegavirales.

Family	Subfamily	Genus	Type Species	Hosts
Bornaviridae		Borna Virus	Borna Disease Virus	Vertebrates
Filoviridae		Marburgvirus	Lake Victoria marburgvirus	Vertebrates
		Ebolavirus	Zaire ebolavirus	Vertebrates
Paramyxoviridae	Paramyxovirinae	Avulavirus	Newcastle disease virus	Vertebrates
		Henipavirus	Hendra virus	Vertebrates
		Morbillivirus	Measles virus	Vertebrates
		Respirovirus	Sendai virus	Vertebrates
		Rubulavirus	Mumps virus	Vertebrates
	Pneumovirinae	Pneumovirus	Human respiratory syncytial virus	Vertebrates
		Metapneumovirus	Avian pneumovirus	Vertebrates
Rhabdoviridae		Vesiculovirus	Vesicular stomatitis Indiana virus	Vertebrates, Invertebrates
		Lyssavirus	Rabies virus	Vertebrates
		Ephemerovirus	Bovine ephemeral fever virus	Vertebrates, Invertebrates
		Novirhabdovirus	Infectious haematopoetic necrosis virus	Vertebrates
		Cytorhabdovirus	Lettuce necrotic yellows virus	Plants, Invertebrates
		Nucleorhabdovirus	Potato yellow dwarf virus	Plants, Invertebrates

2.2.2. Genomic organisation

BDV is an enveloped, non-segmented, single- and negative-stranded RNA virus (NNS RNA). Replication and transcription of the genom take place in the cell nucleus. The genome size is about 8.9 kilo bases, which makes it the smallest among known negative-stranded RNA viruses.

Negative stranded RNA viruses initiate infection by introducing their genetic material in the form of ribonucleoprotein complexes into the host cells. The molecular biology of BDV is complex, and includes overlap of the open reading frames (ORFs) and transcription units, transcriptional read-through of termination signals, and differential use of initiation codons[5, 23]. BDV uses a cellular splicing machinery to generate some of its mRNAs[5, 22].

BDV encodes at least six proteins (fig. 1). Five proteins correspond to the nucleoprotein (N, p40), phosphoprotein (P, p24), matrix protein (M, p16), glycoprotein (G, p57), and L-polymerase (L, p190) found in other Mononegavirales. The sixth, p10 (X protein), does not have a clear homologue in other NNS RNA viral systems[5, 13].

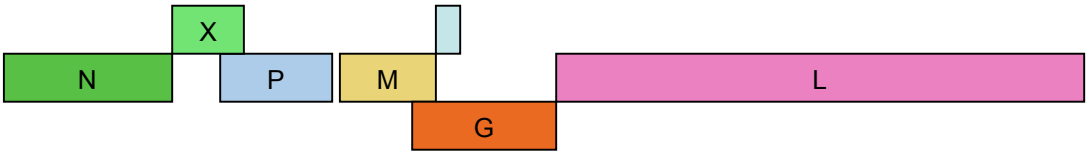

Fig.1. Genomic organisation of BDV.

2.3. Hosts and Epidemiology

BDV infections mainly affect horses and sheep but the disease is not strictly limited to these two hosts, although the incidence in other animals (donkeys, goats, and cattle) appears to be very low. However, the identification of BDV in hosts such as cats, dogs, lynx, rabbits, and even ostriches indicates that the virus has a broad host repertoire of various birds and mammals. Finally, experimental infection with BDV has been demonstrated in many laboratory animals, such as rats, mice, rabbits and gerbils[16]. Many fundamental questions regarding the epidemiology of BDV remain unsolved. Since horizontal transmission of BDV from diseased horses or sheep to uninfected animals does not appear to occur, other sources of infection, i.e. a different natural

reservoir of BDV must exist[9]. In a previous report we had postulated that shrew mice could be candidates for reservoir hosts of BDV[10].

Regarding the seasonal incidence of natural BD cases, fig. 2 shows the distribution of 107 BD cases in Switzerland, which had been diagnosed at the Institute of Veterinary Pathology in Zuerich (47 horses, 46 sheep, 4 donkeys, 2 mules, 3 goats, 2 cows, 2 rabbits and 1 cat) during the last 30 years (1976 until today).
Figure 2 shows a peak during the summer months, but there are as well cases during the other months. An other publication by Ludwig et al. had reported the highest incidence in horses during spring[15]. So the question arised if this appearance would correlate with the occurrence of shrew mice or other possible vector populations. However the incubation time is supposed to be up to several months[4], which might explain BD cases occurring in wintertime.

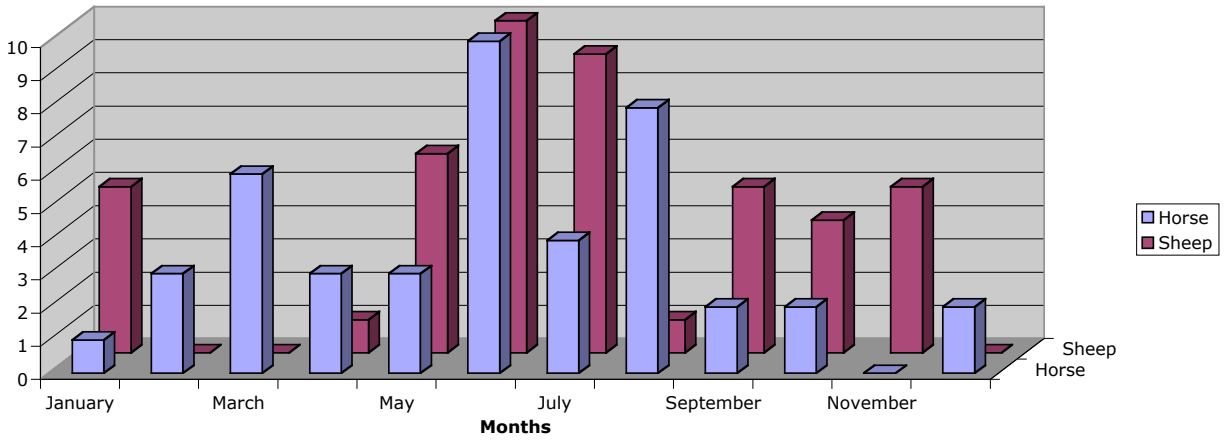

Fig. 2. Annual distribution of BD cases in Switzerland during the last 30 years.

2.4. Clinical and Pathological Manifestations

Borna disease has been described as a progressive nonpurulent meningoencephalomyelitis (fig. 3) of horses and sheep with various clinical symptoms ranging from slightly impaired coordination to paralysis and death[9]. Post mortem changes are largely confined to the gray matter of the CNS and are most severe in the midbrain, midbrain-diencephalon junction, hypothalamus, and hippocampus. Inflammation of the meninges and spinal cord is generally mild. Small, round to oval, eosinophilic intranuclear inclusions occur in neurons of the brain stem, hippocampus, and cerebrospinal ganglia. In the peripheral neuron system, inflammation occurs in the cranial, spinal, and autonomic ganglia and in the peripheral nerves.

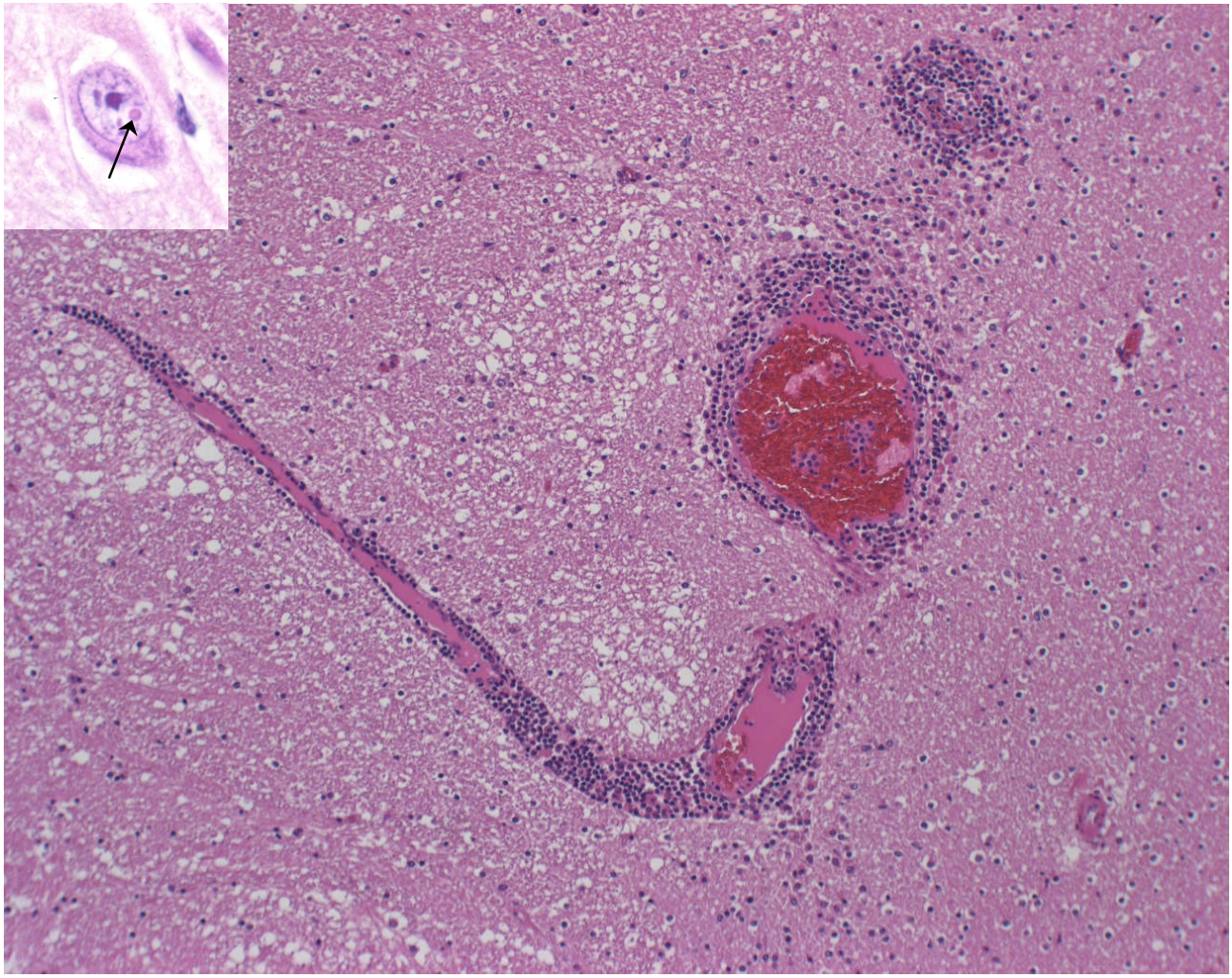

Fig.3. CNS, horse, HE, 10x objective, nonpurulent encephalitis; inset: intranuclear inclusion body in a nerve cell (arrow).

The incubation period in horses and sheep is variable, between two weeks and a few months, probably depending on several factors, i.e. on the site of infection and on the virus strain.

Carbone et al. (1987) could show that experimentally inoculated rats developed encephalitis after an incubation period of 17 to 90 days. Their report shows that the incubation period is the time required for transport of the agent in dendritic-axonal processes from the site of inoculation to the hippocampus. The immune response to the agent had no effect on replication or transport of the virus[4].

During the initial phase, nonspecific signs such as hyperthermia, anorexia, and alternance of colic and constipation are observed. During the acute phase, neurological signs result from meningoencephalitis, such as abnormal posture, ataxia, proprioceptive deficit and repetitive movements. These signs can be associated with abnormal reactions to external stimuli such as hyperexcitability, aggressiveness, lethargy, somnolence and stupor. In the final phase, paralysis can appear, followed by convulsions. Death usually occurs after one to three weeks and the death rate in horses is above 80%, and 50% in sheep. In animals that have survived the acute phase of the disease, recurrent episodes may possibly appear for the rest of the animals life (chronic infection) with depression, apathy, somnolence, fearfulness, in particular after stress. However this is still controversial. Although the clinical pattern is still considered to represent classical BD, infection may also result in asymptomatic carrier status, subtle disturbance in learning and memory, profound disorders of behaviour and movement or death.

Various laboratory animals are susceptible to BDV infection. The incubation period, mortality and severity of the disease considerably depend on the infected animal species, viral variant and host immune status. In adult immunocompetent animals, the infection causes a meningoencephalitis as in horses and sheep. However immunosupressed animals or animals with immature immune system show more discrete symptoms[20].

2.5. Vector candidates

2.5.1. Mice and rats – experimental infections

Earlier studies in rats have shown, that the clinical course and histopathology of Borna disease varies with the age of the animal at the time of infection. In adult Lewis rats, BDV infection results in severe encephalitis accompanied by clinical symptoms that include hyperactivity, aggressiveness, and ataxia. At a later stage of the disease, surviving animals are apathetic and show signs of dementia and behavioral abnormalities, and their brains show a dramatic loss of neuronal tissue[1]. Experimental infection of adult mice takes a nonsymptomatic course, an observation previously believed to indicate that this animal species is not suitable for pathogenesis studies. The group of Peter Staeheli et al.(1998) however demonstrated that BDV frequently induces severe neurological disease in infected newborn mice[8].

2.5.2. Shrews

Shrews belong to the order insectivora (table 2, fig. 4). Their appearance can be described as something between a mouse and a mole. It has inconspicuous eyes and ears and five toes on each foot. It is small (3.5 to 6 cm in wide, 8.9 - 15.2 cm in length), usually greyish or brownish in color, with a lighter color beneath, and both sexes look alike. Shrews are very swift runners above ground.

Favourite habitats are moist forest floors, swamps, marshes, bogs, tundra and mountains, but a few species prefer dry areas. Shrews make their homes in small, round holes in leaf litter around rocks and logs. They use surface paths of mice and voles to move about for food. Shrews are aggressive, irritable, and nervous in nature; their diet consists of insects, earthworms, grubs, other invertebrates and mice, as well as berries and soft vegetation. Many shrews will eat the equivalent of their own body weight every 24 hours.

Shrew’s breed early in the year and many species have more than one litter in a year. Shrew’s gestation period lasts from eighteen to twenty-two days and litters range from four to ten in number, with the young typically being on their own within three weeks of

birth. Shrews normally only live to an age of two years, and they do not hibernate in the winter months.

They utter tiny, high – pitched squeaks to communicate. For non-experts, it is very difficult to distinguish the different species of shrews from their appearance.

Table 2. Taxonomic structure of bicoloured white-toothed shrews

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Class	Mammalia
Order	Insectivora
Family	Soricidae
Subfamily	Crocidurinae
Genus	Crocidura
Species	**Crocidura leucodon,** Crocidura russula, Crocidura suaevolens

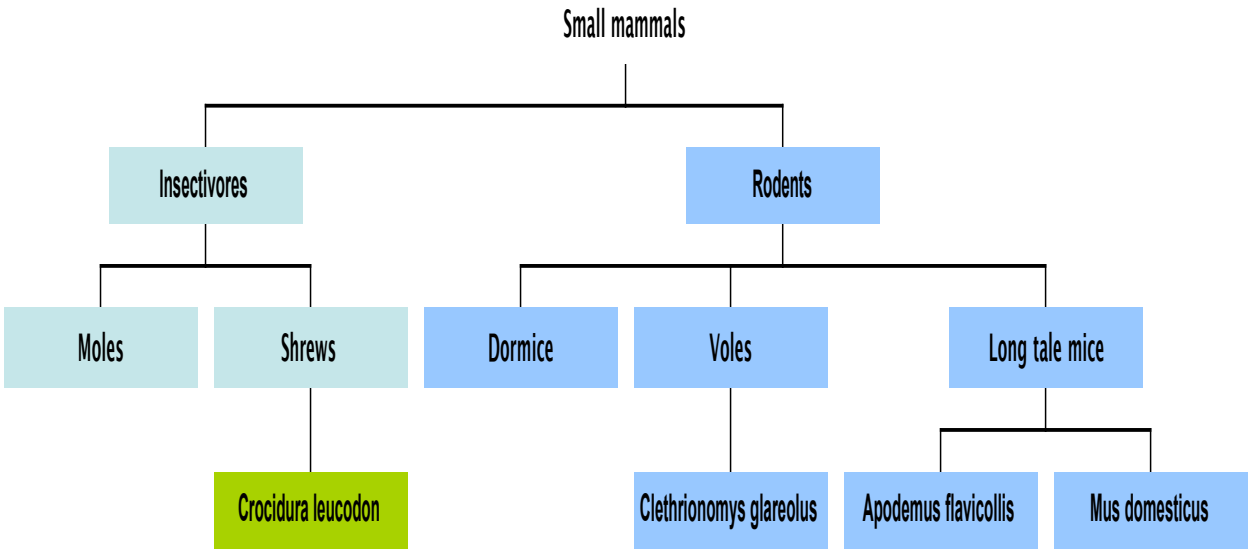

Fig.4. Systematics of small mammals

Crocidura leucodon (bicolored white-toothed shrew):
The bicolored shrew belongs to the genus *Crocidura* together with two other species which occur in Europe (C.russula and C.suaveolens).

Fig.5. Crocidura leucodon (bicolored white-toothed shrew)

- Mid-size shrew (head and body: 29-41mm, weight: 7-13g)
- Fur clearly bicolor; back: dark-grey or brown-grey, belly: white or pale grey.
- Bicolor tail.
- Biology and biotope not well known (they love sunny and warm hills).
- Present in eastern part of CH (Rhine Valley, Tessin), in the Rhône Valley, in the region of Basel, and the Principality of Liechtenstein.

2.5.3. Birds
Berg et al. (2001) were able to amplify fragments of the BDV p24 and p40 genes from faecal samples collected at a bird pond. To their knowledge, this was the first confirmed finding of BDV in wild birds[2].
Recently, Kistler et al. (2008) demonstrated the existence of an avian reservoir of remarkably diverse bornaviruses and provided a compelling candidate in the search for an etiologic agent of proventricular dilation disease (PDD). For almost 40 years, a viral etiology for PDD had been suspected, but no candidate etiologic agent had been reproducibly linked to the disease[12].

2.5.4. Ticks
Ticks had been suspected to be possible vectors for BDV. In order to investigate the ability of ticks to acquire and multiply the BDV, ticks were experimentally fed with a suspension containing infected MDCK cells. The virus load per tick gradually decreased

over time after ingestion of BDV-positive MDCK cells, reaching background levels between 10 and 25 days post infection. This detection was made by using the TaqMan® Real-Time PCR technology[20]. The conclusion was made that ticks can get the infection by ingestion, but since viremia is not a consistent feature in BDV infected mammals, the role of ticks in the transmission of BDV remains uncertain and rather unlikely.

2.5. Aim of the study

After our initial report on BDV infections in *Crocidura leucodon* (bicolored white-toothed shrew) and their possible role as vector species[20], the aim of this investigation was to confirm this observation by collecting and testing additional animals and to define the exact tissue distribution of BDV antigen by means of immunhistochemistry and RT-PCR.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Small mammals

Between 2004 and 2006, 69 mice (2 *Arvicola terrestris*, 46 Apodemus sp., 9 *Clethrionomys glareolus*, 7 *Mus domesticus,* and 5 Microtus sp.) and 12 shrews (6 *Sorex araneus* and 6 *Crocidura leucodon*) were trapped using live traps (Longworth Mammal Trap with NestBox, Penlon Limited, Abingdon, England) around different villages of the endemic area in Graubünden (Fanas, Malans, Malix, Untervaz, Maienfeld, Bonaduz, and Seewis). Two BVD-positive *Crocidura leucodon* were trapped in Seewis, a small village in the Praettigau Valley, located approx. 15 km north-north-east of Chur, Switzerland, at 1000 m above sea level, approx. 20 km apart from the previous trapping area. This region is also considered endemic for BD in horses and sheep.

All places have been selected because of positive BD cases that have arisen in the past. One sheep and four horses were affected in Malix GR, one horse in Fanas GR, and one horse, one mule, and two sheep in Malans GR.

Fig. 6. Map of Malix GR, Fanas GR and Malans GR

Fig. 7. The pasture in Fanas GR; typical biotope for the occurence of C.leucodon

3.2. Methods

3.2.1. Histology

The small mammals, which were captured, were humanely euthanized and dissected within 24 hours. Their brains were divided into 2 equal parts, one half was fixed in 4% formaldehyde, cut longitudinally into several equal parts and embedded in paraffin for microscopic evaluation the other half was stored in tubes at –20°C. Likewise, half of heart, lung, liver, intestinal tract, kidney, genital tract, skin, salivary gland, adrenal gland, and a longitudinal section through the skull were fixed in 4% formaldehyde and embedded in paraffin for microscopic evaluation, while the other half of the above-mentioned organs and tissues were stored frozen at –20°C.

3.2.2. Immunohistochemistry for BDV antigen

BDV antigen staining was achieved employing two different monoclonal antibodies directed against the p24 and p38/40 proteins of BDV, respectively (gift from Prof. L. Stitz, FLI Tübingen, Germany).

Formalin fixed and paraffin embedded sections were deparaffinized and rehydrated in xylene and descending alcohol. Sections were then counterstained with haemalaun for 2 min and watered for 3 to 5 min. After digestion with 0,1% Pronase (DAKO Cytomation, Copenhagen, Denmark, S2013) for 10 min at room temperature, endogenous peroxidase was blocked by incubation with H_2O_2 (3% in H_2O + 0,2% in NaN_3) for 10 min at room temperature. Sections were incubated with two monoclonal mouse-anti BDV antibody, which recognises p38/40 and p24 (L. Stitz, Tübingen, Germany) in a dilution of 1:500 over night at room temperature. Then sections were incubated for 15 min at room temperature with a secondary anti-mouse/anti-rabbit-biotinylated antibody (ChemMate^{TM}-KIT, DAKO Cytomation, Copenhagen, Denmark, K5003). Finally, streptavidin-peroxidase (ChemMate^{TM}-KIT, DAKO Cytomation, Copenhagen, Denmark, K5003) was added for 15 min at room temperature. After each step slides were thoroughly washed with PBS (Phosphate buffer solution, pH 8.0). Finally, the reaction was visualised with AEC substrate (AEC-Substrate chromogen kit, Zymed, San Francisco, CA, USA, 00-2007).

3.2.3. Immunohistochemical markers for cell type identification

For exact identification of infected cells, the following cell markers were used: GFAP (DAKO, Cytomation, Zug, Switzerland) for astrocytes, CNPase (Chemicon International) for oligodendrocytes, NFP (DAKO, Cytomation, Zug, Switzerland) for axons, MAP2 (Chemicon International) for myelin sheaths, and actin (DAKO, Cytomation, Zug, Switzerland) for smooth muscle cells.

3.2.4. PCR amplification by TaqMan® technology

PCR amplification and quantification were performed on ABI 7700 real-time Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland)[21].

3.2.4.1. RNA extraction and reverse transcription

All tissue samples (30 mg per 40 µl) were disrupted and homogenized with QIAshredder, and total RNA was subsequently isolated using the QIAamp® Viral RNA Mini Kit (for skin and urine, the latter collected on blotting paper) and the RNeasy® Mini Kit (QIAGEN AG Basel, Switzerland; for all other tissue samples), respectively.

RNA from all samples was then digested with 1 µl Rnase free Dnase (Promega, Dubendorf, Switzerland) for 1h at 37°C, followed by a deactivation step at 95°C for 5 min.

Isolated and purified RNA was reverse transcribed into cDNA using the Reverse Transcriptase System (Promega, Dubendorf, Switzerland). Briefly, total RNA was incubated with random primer and 30 units RT for 1h at 42°C. Then the RT was inactivated for 5 min at 95°C. The cDNA was stored at –20°C until further use.

3.2.4.2. Principles

The basis for PCR quantitation in the ABI 7700 system is to continuously measure PCR product accumulation using a dual-labeled fluorogenic oligonucleotid probe called a TaqMan® probe. This probe is composed of a short (ca. 20-25 bases) oligodeoxynucleotide that is labeled with two different fluorescent dyes. On the 5' terminus is a reporter dye (FAM) and on the 3' terminus is a quenching dye (TAMRA). This oligonucleotide probe sequence is homologous to an internal target sequence

present in the PCR amplicon. When the probe is intact, energy transfer occurs between the two fluorophores and the quencher suppresses emission of the reporter. During the extension phase of PCR, the probe is cleaved by 5' nuclease of Taq polymerase thereby releasing the reporter from the oligonucleotid-quencher and producing an increase in reporter emission intensity (fig. 8). The ABI Prism 7700 uses fiber optic systems, which connect to each well in a 96-well PCR tray format. The laser light source excites each well and a CCD camera measures the fluorescence spectrum and intensity from each well to generate real-time data during PCR amplification. The ABI 7700 Prism software examines the fluorescence intensity of reporter and quencher dyes and calculates the increase in normalised reporter emission intensity over the course of the amplification. The results are then plotted versus time, represented by cycle number, to produce a measure of PCR amplification. To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined at a point during the early log phase of product accumulation. This is accomplished by assigning a fluorescence threshold above background and determining the time point at which each sample's amplification reaches the threshold (defined as the threshold cycle number or C_T). Differences in threshold cycle number are used to quantify the relative amount of PCR target contained within each tube as described previously.

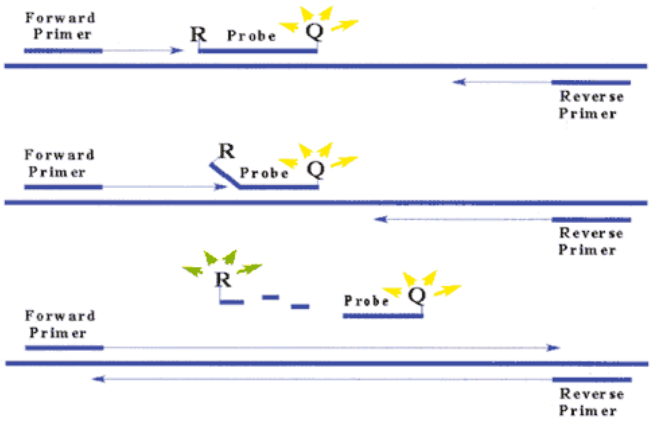

Fig. 8. Scheme of the TaqMan® technology(TaqMan® Universal PCR Master Mix, Protocol, Applied Biosystems, Foster City, CA, USA).

3.2.4.3. Reaction conditions

PCR amplification was carried out in 25µl reaction mixtures per well containing 12.5µl of 2x TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 600nM of each primer, 80nM (p40-56T) and 160nM (p24-54T) of the probes, and 3.5µl of cDNA. In addition, 18s rRNA was amplified as an internal control to monitor the quality of the extracted RNA (PE Biosystems, Warrington, UK). The same 18s primers/probe were used for amplification of cDNA from the brain tissue and the organs. PCR conditions were set as follows: 2 min at 50°C to activate the Uracil-N-Glycosilase, which detects possible former PCR products and destroys them, and then 10 min at 95°C to inactivate the Uracil-N-Glycosilase and activate the polymerase followed by 40 cycles consisting of denaturation at 95°C for 15s and annealing and elongation at 60°C for 1 min. The data were analysed with the sequence detector software (version 1.7). Signals were regarded positive if the fluorescence intensity (increase of fluorescence ΔR_n) exceeded 10 times the standard deviation of the baseline fluorescence (threshold cycle [C_T]). C_T values of 40 were regarded as negative. The threshold cycle or C_T value is the cycle at which a statistically significant increase in ΔR_n is first detected[20].

3.2.5. Conventional RT-PCR

Selected frozen-stored parallel samples including brain specimens from all animals which proved BDV-positive in the Zurich laboratory as well as a brain specimen of a BDV-positive sheep from the same pasture where the small mammals were trapped were sent to the Vienna laboratory for independent confirmation by conventional RT-PCR and subsequent sequencing of the amplification products. RT-PCR and sequencing were carried out as described by Kolodziejek et al.[14]. The resulting amplicons were sequenced by employing the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA) and an ABI Prism 310 genetic analyzer (PE Applied Biosystems).

4. RESULTS

4.1. Small Mammals

4.1.1. Histological examination

None of the mice or shrews caught in the endemic areas mentioned showed any histological changes, which would be typical for Borna disease, such as disseminated mononuclear meningitis and polioencephalomyelitis with subsequent neuronal degeneration and eosinophilic intranuclear inclusion bodies (Joest-Degen inclusion bodies). Moreover, no microsopic lesions were found in any of the other organs investigated.

4.1.2. Immunohistochemistry

With the exception of *Crocidura leucodon* all other species of small mammals trapped and analyzed during this study proved negative for BDV antigen and nucleic acid – including 7 *Sorex araneus* shrews. From 6 *Crocidura leucodon* shrews, however, two (nos. 67 and 68) turned out to be BDV-positive (one adult female and one adult male). These shrews were trapped in August 2006, i.e. more than 3.5 years later and approx. 20 km apart from the place where 3 other *Crocidura leucodon* shrews were BDV-positive[10].

In the two shrews BDV antigen was detected widespread and with intense signals by IHC using the two monoclonal antibodies p24 and p38/40, respectively. A similar staining pattern was observed with both antibodies, although the signal was stronger using the p38/40 antibody. Several organs, including brain, heart, lung, liver, intestinal tract, kidney, genital tract, skin, and a longitudinal section through the skull showed labeling of small intranuclear inclusion bodies and, in addition, some intracytoplasmatic staining was noted, mainly in nerve tissue as well as in epithelial and parenchymal cells within the various organs (fig. 9-11). Demonstration of Borna disease virus (BDV) p24 antigen in tissues of bicolored white-toothed shrews (*Crocidura leucodon*) by immunohistochemistry (ChemMate method [DAKO, Cytomation, Zug, Switzerland]).

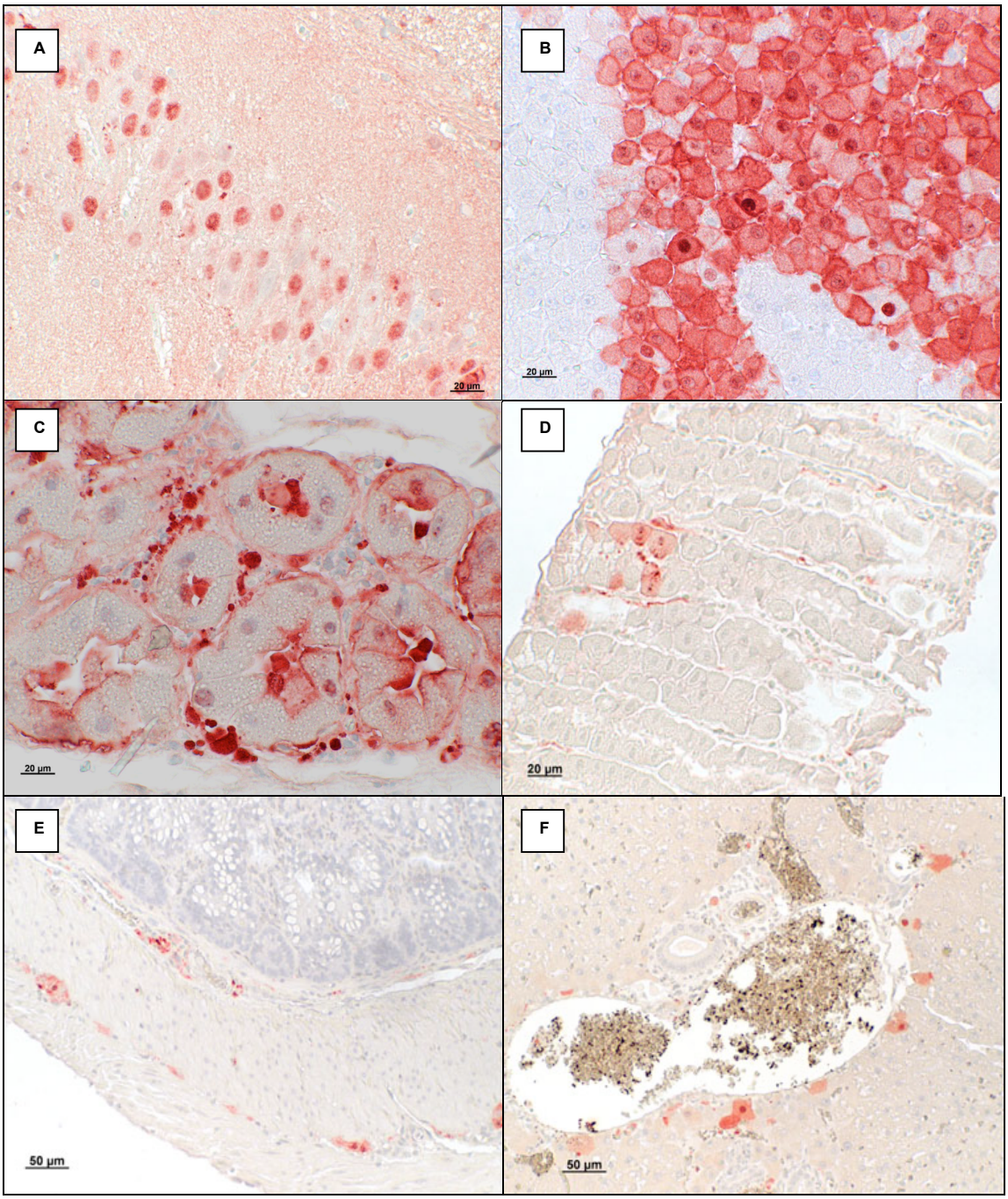

Fig. 9 A-F. BDV antigen detection (p38) by immunohistochemistry in tissue paraffin sections (shrew 67): A) Brain: BDV antigen in the hippocampus. Note the so-called Joest-Degen intranuclear inclusion bodies in multiple neurons of the gyrus dentatus; in some neurons a homogenous intracytoplasmatic staining can be seen B) Adrenal gland: homogenous intracytoplasmatic staining can be seen in most of the medullary cells. C) Salivary gland: labeling of some of the nuclei of the glandular epithelium and of nerve fibers surrounding the glands, as well as of the apocrine secretion. D) Gastric mucosa: BDV antigen in zymogenic cells; homogenous intracytoplasmatic staining and intranuclear inclusion bodies can be seen in some of the cells. E) Intestine: BDV antigen in the plexus myentericus and submucosus. F) Hepatocytes with homogeneous intracytoplasmatic and intranuclear labeling.

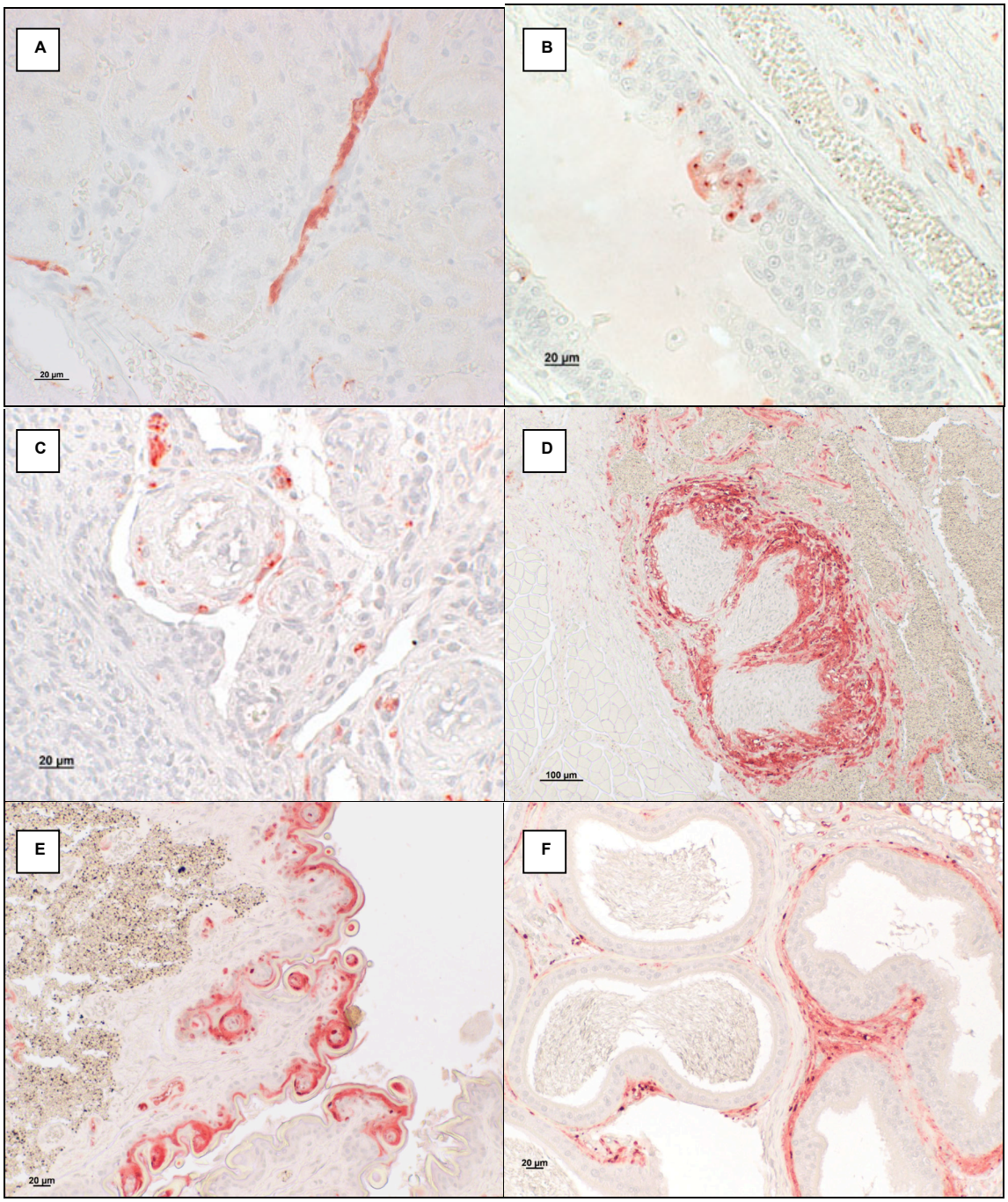

Fig. 10 A-F. BDV antigen detection (p38) by immunohistochemistry in tissue paraffin sections of the urogenital tract of shrew no 67 (male) and no 68 (female):

A) Kidney: nerve fibers along the tubules are specifically stained. B) Urethra (pars penis): BDV antigen in the transitional epithelium (shrew no 67): note the homogenous intracytoplasmatic staining and the intranuclear inclusion bodies. C) Uterus: nerve fibers surrounding blood vessels show positive labeling (shrew no 68). D) BVD antigen in the cavernous body of the penis; mainly nerve fibers are stained (shrew no 67). E) BDV antigen in the epithelium of the glans penis: epithelial cells as well as subepithelial nerve fibers are labeled (shrew no 67). F) Epididymis: mainly the nerve fibers around the seminiferous tubules are stained (shrew no 67).

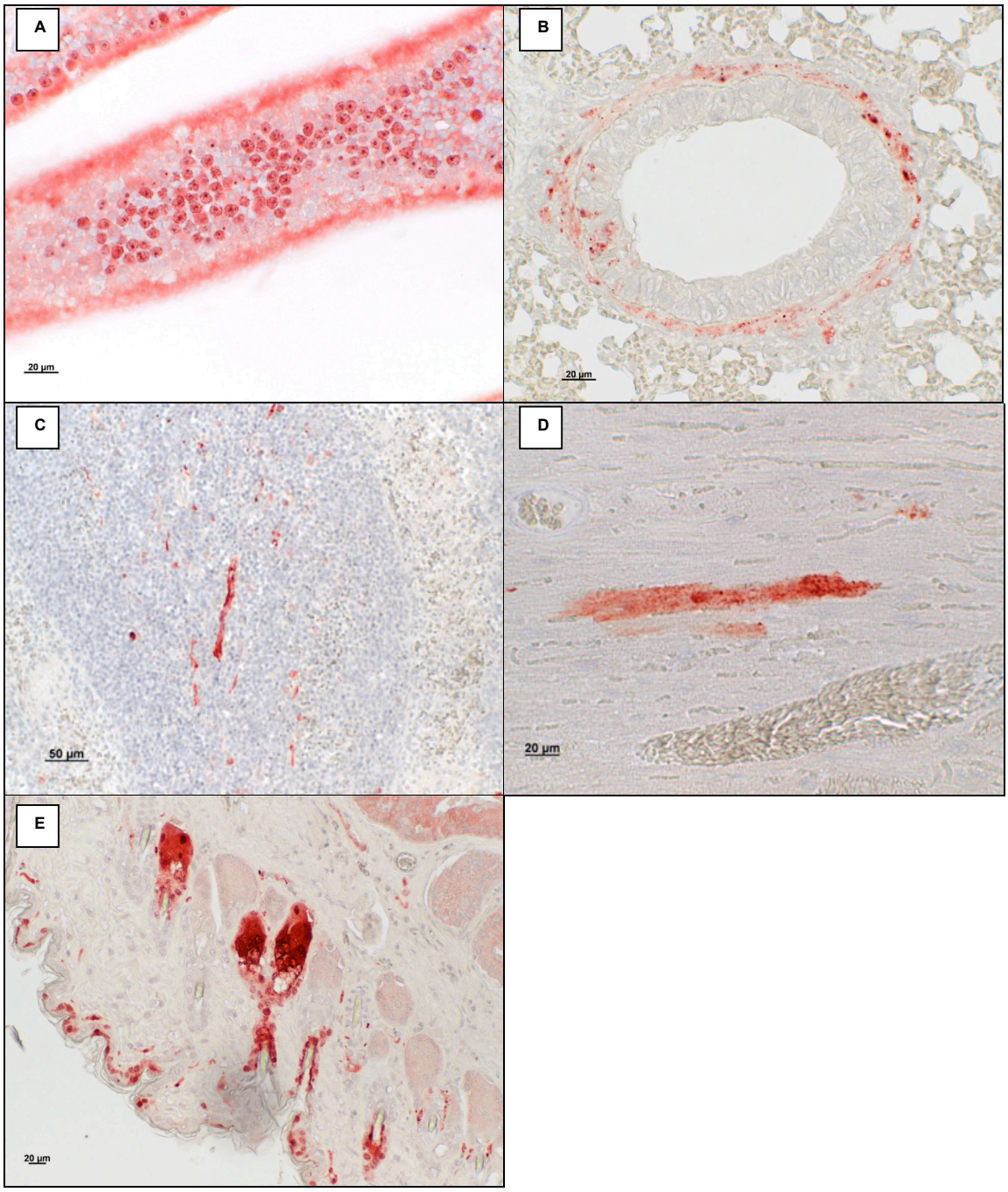

Fig. 11 A-E. BDV antigen detection (p38) by immunohistochemistry in tissue paraffin sections of shrew no 67:

A) Respiratory epithelium in the nose. Note the homogenous intracytoplasmatic staining and the intranuclear inclusion bodies. B) Peribronchiolar nerve tissue and some bronchiolar epithelial cells are positive. C) Spleen: nerve fibers within the follicle are specifically stained.

D) Myocardium: positive staining of a single myocardial fiber. E) Skin: strong staining of sebaceous glands and epithelial basal cells.

BDV antigen was detected in cerebrum, cerebellum, and in the optic nerve, mainly in neurons, but also in oligodendrocytes, astrocytes, and in Purkinje cells: distinct labeling of the nuclei was observed, and some of the cells showed positive staining of Joest-Degen intranuclear inclusion bodies. Furthermore a slight intracytoplasmatic staining was seen.

Different other organs including spleen, lung, liver, heart, kidney, salivary gland, skin, penis, testis, epididymis, stomach, intestine, urinary bladder, and uterus showed a diffuse labeling of small nerves within the parenchyma and around blood vessels. Virus antigen was detected within some parenchymal cells (tubular cells) of the kidneys, some hepatocytes in the trias, some Leydig cells, single myocardial cells, and within some epithelial cells like epidermis, salivary glands including saliva, sebaceous glands, transitional epithelium of the urethra, and bronchiolar epithelium. Some epithelial cells of bronchioles in the lungs, tubular cells in the kidneys, epithelial cells of the epidermis, penis, and epithelial cells of the salivary glands including the secretion showed either a distinct nuclear or a slight diffuse intracytoplasmatic labeling. A few hepatocytes within the portal area of the liver, myocardial fibres in the heart, peptic cells of the stomach, and Leydig cells in the testis exhibited an intranuclear and/or diffuse intracytoplasmatic staining. Nerve tissue within the intestinal wall, the cavernous body of the penis, the spleen and epididymis showed distinct either diffuse or granular staining. Antigen was also detected in small nerves around blood vessels within the uterus and mamma. The adrenal gland exhibited a very strong intracytoplasmatic and nuclear labeling of the medullary cells (table 3).

Table 3. Immunohistochemical detection of Borna disease virus (BDV) antigen in various organs.

Organ	Shrew no. 67 (male)	Shrew no. 68 (female)	Comments
Brain	+++	+++	Neurons (c., n.), Purkinje cells (c., n.)
Eye	+	+	Optic nerve (c.)
Skull	+++	+++	Respiratory epithelium (c., n.), nerve tissue (c.)
Heart	+	+	Muscle fibers (c., n.)
Spleen	++	++	Nerve tissue (c.)
Liver	++	++	Hepatocytes (c., n.)
Kidney	+	+	Tubular epithelial cells (c., n.), nerve tissue (c.)
Lung	+	+	Bronchiolar epithelial cells (c., n.), nerve tissue (c.)
Adrenal gland	+++	+++	Medullary cells (c., n.)
Salivary gland	++	++	Myoepithelial cells (c.), nerves (c.), glandular cells (c., n.), secretion
Skin	+++	+++	Sebaceous glands (c., n.), epidermal cells (c., n.)
Urinary bladder	+	+	Epithelial cells (c., n.)
Penis	+++		Nerve tissue within the cavernous body (c.), epithelial cells of the glans penis (c., n.)
Testis	+		Some Leydig cells (c., n.)
Epididymis	++		Nerve tissue (c.), myoepithelial cells around the ducts (c., n.)
Gonadal gland	+		Nerve tissue (c.)
Uterus		+	Nerve fibres (c.), myoepithelial cells (c., n.)
Mammary gland		-	
Ovary		-	
Stomach	+	+	Few peptic cells (c., n.)
Intestine	+	+	Plexus myentericus (c., n.), plexus submucosus (c., n.)

c.: cytoplasmatic labeling; n.: nuclear labeling; -: negative; +,++,+++: degree of positivity

4.1.3. PCR amplification by TaqMan® technology

Positive results were also obtained by TaqMan real-time RT-PCR; there was a clear correlation of the intensity of staining seen in IHC and the amount of viral RNA in the visceral organs and brain (table 4), but not in the skin.

Urine was collected from the shrews by using a blotting paper on the bottom of the live traps, and subsequently examined by TaqMan® real-time RT-PCR. The urine samples contained cells, based on the 18s rRNA values, but no virus RNA.

*Table 4. Summary of immunohistological and real-time RT-PCR results.**

Shrew no.	Organ	Immunohistology	Real-time RT-PCR Ct values† p24 and p40 (2 analyses)	Real-time RT-PCR Ct values 18s rRNA (mean values)	Calibrated values‡ (nucleic acid copies; mean of 2 analyses)
67	Skin§	p24 +++ p40 +++	p24: 30.3/30.2 p40: 31.6/32.1	40.00	p24: neg p40: neg
	Brain	p24 +++ p40 +++	p24: 20.5/20.6 p40: 20.4/21.3	18.11	p24: 294.9 p40: 131.4
	Kidney	p24 + p40 +	p24: 35.9/35.0 p40: 34.2/34.7	28.44	p24: 11.2 p40: 18.4
	Liver	p24 + p40 +	p24: 34.2/33.9 p40: 34.1/34.6	23.51	p24: 0.8 p40: 0.6
	Lung	p24 + p40 +	p24: 26.5/26.6 p40: 27.2/27.4	19.46	p24: 10.2 p40: 3.8
	Heart	p24 + p40 +	p24: 27.7/28.1 p40: 27.5/28.3	17.05	p24: 0.7 p40: 0.5
	Urine§¶	n.d.	p24: 40.0/40.0 p40: 40.0/40.0	36.84	p24: neg p40: neg
68	Skin§	p24 +++ p40 +++	p24: 34.6/34.1 p40: 37.3/36.0	40.00	p24: neg p40: neg
	Brain	p24 +++ p40 +++	p24: 25.0/24.6 p40: 25.2/25.5	22.42	p24: 311.1 p40: 125.7
	Kidney	p24 + p40 +	p24: 31.0/31.3 p40: 31.5/31.1	24.77	p24: 17.1 p40: 11.3
	Liver	p24 + p40 +	p24: 37.2/38.3 p40: 36.9/37.8	24.49	p24: 0.1 p40: 0.2
	Lung	p24 + p40 +	p24: 30.5/30.5 p40: 32.0/31.9	21.23	p24: 2.1 p40: 0.6
	Heart	p24 + p40 +	p24: 27.4/27.4 p40: 27.9/28.2	18.65	p24: 3.0 p40: 1.3
	Urine§¶	n.d.	p24: 40.0/40.0 p40: 40.0/40.0	24.00	p24: neg p40: neg

**No histopathological lesions were found in any of these organs of both shrews*

†RT-PCR, reverse transcription-polymerase chain reaction; Ct, threshold cycle no.

‡The calibrated values were calculated by using the sum of both normalized Ct-values of Borna disease virus p24 and p40, respectively, and dividing it by the normalized value

of the 18s rRNA. Each organ was analyzed twice, and the evaluation was performed in duplicate.
§QIAamp Viral RNA Mini Kit (QIAGEN AG Basel, Switzerland) was used instead of RNeasy Mini Kit (QIAGEN AG Basel, Switzerland)
¶Urine was collected on a blotting paper on the bottom of the live traps
+++ strong positivity; + slight positivity

4.1.4. Conventional RT-PCR

The TaqMan real-time RT-PCR results were confirmed by conventional RT-PCR[14]. The compiled BDV sequences derived from the two *Crocidura leucodon* shrews as well as the sequence derived from a sheep originating from the same pasture as the shrews resulted in a stretch of 1824 nucleotides (nt), composing the complete N-, X- and P-protein-encoding regions, as well as the 5′-untranslated region of the X/P transcript of the BDV genome. All sequences turned out to be unique, however closely related to each other. The BDV sequence derived from the sheep (GenBank accession no. EU095836) differed from the sequence derived from shrew no. 68 (GenBank accession number EU095835) by 3 nt and from the sequence derived from shrew no. 67 (GenBank accession number EU095834) by 9 nt. Shrews no. 67 and no. 68 showed an 8 nt difference. The sequence of shrew no. 67 exhibited 99.8% identity to a BDV sequence (GenBank accession no. AY374550) derived from a horse that died of BD, and originated from Jenins (Graubuenden, Switzerland, close to Seewis, where the shrews were trapped), and 99.7% identity to a BDV sequence (GenBank accession no. AY374544) derived from another horse from Malans (Graubuenden, Switzerland), which died of BD near the location where the shrews were trapped. And finally, the sequences of shrew no. 68 and of the sheep showed 99.9% identity to a BDV sequence (GenBank accession no. AY374549) derived from a sheep, which originated from a village named St. Peter (again Graubuenden, Switzerland).

5. DISCUSSION

In contrast to the distribution of BDV antigen and RNA in horses and sheep suffering from classical BD, *Crocidura leucodon* shrews harbor BDV in most of the tissues examined, without evidence of pathological lesions. These findings support the concept that shrews of the species *Crocidura leucodon* are important reservoirs and vectors of BDV, as postulated in a previous publication[10]. Up to now, we did not find BDV antigen or RNA in any of the other small mammals captured including mice and other shrew species. The pathogenesis of classical BD is immune-mediated, the absence of inflammatory lesions points towards immune tolerance or immune suppression. Experimental investigations demonstrated that infection of neonatal rats – having an immature immune system – with BDV results in persistent infection. By intracerebral infection of 1-day-old Wistar rats no clinical signs became evident and these rats did not produce BDV-specific antibodies, while animals inoculated at 1 or 2 months of age showed inflammation in the brain; the latter rats were able to produce anti-BDV antibodies[24, 25]. Another study confirmed the age-dependent differences in the tissue distribution of BDV in rats infected intracerebrally[11].
From experimental BDV infections it is known that there are considerable species-specific differences regarding pathogenesis of the disease. A major difference between the well-established BDV experimental rat model and the mouse model[8, 9, 24] is that infection of adults induces neurological disease in rats but not in mice. While infection of newborn rats leads to immunological tolerance and almost disease-free persistent infection, neonatal infection of mice induces severe neurological disease[9].

Consistent with BDV infection in neonatal rats, *Crocidura leucodon* shrews show a widespread infection without evidence of pathological lesions. Mild degenerative lesions in the cerebellar cortex and in the hippocampus with loss of neurons have been reported in neonatally infected rats[6, 26] which could not be seen in the shrew's brains. The question if shrews are also devoid of BDV-specific antibodies could not be answered so far, due to the very limited amount of blood available post mortem.

Therefore the questions arises if shrews are really immunotolerant towards BDV and at which age shrews will most likely be infected in order to develop a persistent infection, which allow them to survive the infection without clinical signs and without

inflammatory lesions in the CNS or in other organs. An intrauterine infection appears to be possible, based on the presence of viral antigen in reproductive organs such as uterus. The presence of BDV antigen in testis and epididymis are further arguments which point towards the possibility of a vertical transmission. Vertical Transmission of BDV has been reported in a pregnant mare and its foal[7] as well as in experimentally infected mice[17]. On the other hand, infection in the perinatal age is also conceivable in analogy to neonatally infected rats. Immune tolerance would imply absence of specific immune reactions such as humoral antibody production. This question remains open, since we were not able to collect enough blood for testing for BDV specific antibodies.

Another unsolved question is the way of transmission from shrews to other susceptible hosts such as horses and sheep. A possible way could be via excretions and secretions such as urine, saliva or nasal secretions. In *Crocidura leucodon* shrews we could detect BDV antigen and nucleic acid in organs and tissues like urinary bladder, salivary glands and respiratory epithelium, which leads us to the conclusion that BDV is excreted and secreted by these shrews. BDV antigen, by immunohistochemistry, was also present in considerable amounts in skin structures, mainly sebaceous glands and deep epidermal cells. The low recovery rate of BDV RNA by RT-PCR from skin tissue was possibly due to the fact that, based on the 18s rRNA RT-PCR, practically no cells were extracted from skin tissue. Possibly, the extraction kit used was not effective enough for skin tissue. The port of entry of BDV in final hosts such as horses and sheep is generally thought to be the olfactory system, most likely through contact with contaminated feed. Although indirect experimental evidence suggests this route, proof of this hypothesis is lacking[19].

In summary, the findings presented here support the hypothesis that the shrew species *Crocidura leucodon* plays an important role as reservoir and vector species of BDV. The exact way of acquisition and transmission of the infection remains to be investigated.

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